Antimicrobial Resistance

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# Adjuvants Based on Hybrid Antibiotics Overcome Resistance in *Pseudomonas aeruginosa* and Enhance Fluoroquinolone Efficacy

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***Abstract:*** *The use of adjuvants that rescue antibiotics against multidrug-resistant (MDR) pathogens is a promising combination strategy for overcoming bacterial resistance. While the combination of β-lactam antibiotics and β-lactamase inhibitors has been successful in restoring antibacterial efficacy in MDR bacteria, the use of adjuvants to restore fluoroquinolone efficacy in MDR Gram-negative pathogens has been challenging. We describe tobramycin–ciprofloxacin hybrid adjuvants that rescue the activity of fluoroquinolone antibiotics against MDR and extremely drug-resistant Pseudomonas aeruginosa isolates in vitro and enhance fluoroquinolone efficacy in vivo. Structure–activity studies reveal that the presence of both tobramycin and ciprofloxacin, which are separated by a C12 tether, is critical for the function of the adjuvant. Mechanistic studies indicate that the antibacterial modes of ciprofloxacin are retained while the role of tobramycin is limited to destabilization of the outer membrane in the hybrid.*

*P*seudomonas aeruginosa is the leading cause of nosocomial infections and chronic lung infections in cystic fibrosis patients, with mortality rates ranging from 30–50 %.[1] Among Gram-negative pathogens, infections caused by *P. aeruginosa* are particularly difficult to treat since the organism is both intrinsically resistant and capable of acquiring resistance to most antibiotics. This is in large part the result of the low permeability of its outer membrane, which is 12–100 times less permeable than that of *E. coli* owing to its selective porins.[2] The reduced penetration of antibiotics across the outer membrane in *P. aeruginosa* enables secondary adaptive resistance mechanisms to function more efficiently. These mechanisms include efflux as a result of intrinsic or induced expression of efflux pumps, and antibiotic-modifying enzymes.[2] Clinically useful anti-pseudomonal antibiotics are limited to select penicillins (e.g., piperacillin/tazobactam), cephalosporins (e.g., ceftazidime), carbapenems (e.g.,. imipenem), fluoroquinolones (e.g.,. ciprofloxacin), aminoglycosides (e.g., tobramycin), and colistin, but resistance to these agents is steadily increasing and there are no new novel anti-pseudomonal agents in clinical development.[3] Among the aminoglycosides and fluoroquinolones, tobramycin and ciprofloxacin are the most potent agents in their respective classes against *P. aeruginosa*. Both, tobramycin and ciprofloxacin interact with multiple targets and possess distinct uptake mechanisms. The mode of action of tobramycin is concentration dependent. At low concentrations ($\leq 4\ \mu g\,mL^{-1}$), tobramycin binds to the 30S ribosomal subunit, thereby leading to the disruption of protein synthesis, while at higher concentrations ($\geq 8\ \mu g\,mL^{-1}$), disruption of the outer membrane is observed.[4] In contrast, the antimicrobial action of the fluoroquinolones is mediated by inhibition of two type II DNA topoisomerase enzymes, DNA gyrase and topoisomerase IV, which play an essential role in DNA relaxation, the partitioning of replicated chromosomal DNA during cell division, and decatenation reactions.[5] Besides their distinct modes of action, tobramycin and ciprofloxacin also display different uptake mechanisms. The uptake of aminoglycosides and amphiphilic aminoglycosides[6–9] into Gram-negative bacteria involves interactions at sites at which divalent cations cross-bridge adjacent polyanionic lipopolysaccharide (LPS), which causes destabilization of the outer membrane and results in self-promoted uptake of the antibiotic or other extracellular molecules.[10] In contrast, the uptake of fluoroquinolones into Gram-negative bacteria does not occur by self-promoted uptake, but rather involves diffusion across the cell membrane with penetration through the porin pathway.[11]

The distinct uptake pathways of tobramycin and ciprofloxacin provide an opportunity to optimize the transport of fluoroquinolone antibiotics across the outer membrane. We hypothesized that appending a tobramycin-based vector to ciprofloxacin would enable fluoroquinolones (such as ciprofloxacin or moxifloxacin) to penetrate the outer membrane of *P. aeruginosa* through self-promoted uptake. Ideally, this approach should be combined with the original uptake mechanisms of ciprofloxacin to maximize penetration across the outer membrane. To probe this hypothesis, we prepared the tobramycin–ciprofloxacin hybrids **1a**, **1b**, **1c**, **1d**, and **1e**, in which the two drugs are conjoined by a flexible alkyl tether (Figure 1, also see Scheme S1 in the Supporting Information).

The design of tobramycin–ciprofloxacin hybrids was guided by previous structure–activity studies. The C-5 position of tobramycin was selected for attachment of the tether

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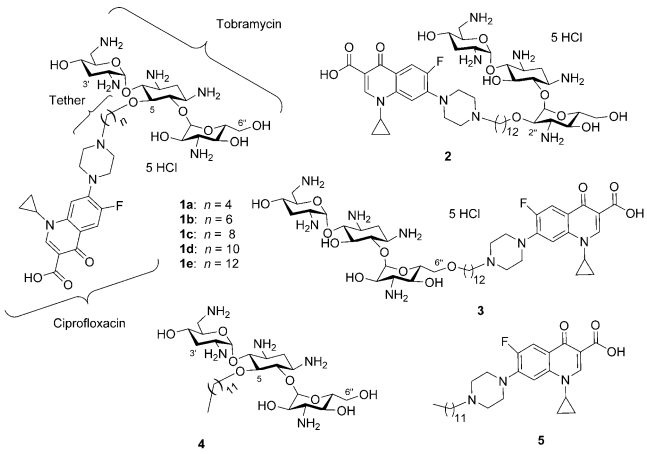


***Figure 1.*** Structures of the tobramycin–ciprofloxacin hybrids. Compounds **1a**–**1e** are hybrids with the tether attached to the C-5 position of tobramycin, while compounds **2** and **3** contain the tether at the C-2″ or C-6″ positions, respectively. Compounds **4** and **5** are fragments of lead structure **1e**.

since this position retains antibacterial activity in Gram-negative bacteria.[12,13] The secondary amino group of the piperazine ring in ciprofloxacin was selected as the point of attachment because it does not interfere with activity against gyrase A and topoisomerase IV in *E. coli* and enhances uptake into Gram-negative pathogens.[5,14] The hybrids were tested for antibacterial activity by determining the minimal inhibitory concentration (MIC) against select Gram-negative and Gram-positive bacteria. Most of the hybrids displayed weak antibacterial activity ($MIC \geq 16\ \mu g\,mL^{-1}$) against the tested bacteria, with the exception of *E. coli* ATCC 25922 ($MIC \geq 2\ \mu g\,mL^{-1}$).In addition, we noticed that **1e** displayed potent antibacterial activity ($MIC = 4\ \mu g\,mL^{-1}$) against a wild-type *P. aeruginosa* strain (Supporting Information Table S1).

To assess whether transport of the fluoroquinolone antibiotics proceeds through multiple uptake mechanisms, we used *P. aeruginosa* PAO1 to determine whether combinations of hybrids **1a**–**1e** with moxifloxacin (Figure 2a) or levofloxacin (Table S2) were additive or synergistic by using the fractional inhibitory concentration (FIC) index[15] as a measure of the interaction between two antimicrobial agents. FIC indices of 1–2, $\leq 0.5$, and $\geq 4$ indicate no interaction, synergy, and antagonism, respectively.[16] The results indicate that hybrid **1e** demonstrates optimal synergy (FIC index 0.077–0.139) in terms of growth inhibition. Synergism was also observed with **1b**, **1c**, and **1d** but not with **1a**, tobramycin, ciprofloxacin, hybrid fragment tobramycin–C12 (**3**), or hybrid fragment ciprofloxacin–C12 (**4**), thus indicating that the dual function of the two antibiotics is required for synergy (Table S2).

Hybrid **1e** was selected for further combination studies against a panel of 8 clinical *P. aeruginosa* isolates, including six MDR (non-susceptible or resistant to $\geq$ 3 chemically unrelated anti-pseudomonal classes), and six extremely-drug resistant (XDR; non-susceptible or resistant to $\geq 5$ chemically unrelated anti-pseudomonal classes; Table S3) isolates. The panel also included two *P. aeruginosa* strains that are non-susceptible or resistant to colistin. The fluoroquinolone-resistant isolates contained a single mutation (T83-I) in GyrA, which was absent in the strains that are non-susceptible or resistant to colistin (Table S4). We determined the FIC index of **1e** in combination with moxifloxacin or ciprofloxacin against six colistin-susceptible XDR *P. aeruginosa* strains. The results indicated that **1e** displays strong synergy, with FIC indices of 0.03–0.28 against these pathogens (Figure 2b and Table S5). More importantly, ciprofloxacin-susceptible ($MIC = 1\ \mu g\,mL^{-1}$) or intermediate resistant ($MIC = 2\ \mu g\,mL^{-1}$) CLSI breakpoints were reached for 6/7 ciprofloxacin-resistant, MDR, or XDR *P. aeruginosa* isolates at **1e** ($< 6.5\ \mu M$). In comparison, the same susceptible and intermediate resistant breakpoints were reached for moxifloxacin in 5/8 moxifloxacin-resistant, MDR, or XDR *P.aeruginosa* isolates (Table S5). As a control, combination studies of moxifloxacin or ciprofloxacin with tobramycin were performed and the results indicated no synergistic effects against XDR *P. aeruginosa* strains (Table S6). Similarly, combination studies of moxifloxacin with known permeabilizers[17] of *P. aeruginosa*, including colistin and cetrimonium bromide but also cationic amphiphiles such as benzethonium chloride and lipopeptide C16-(Dab)4-$NH_2$, against XDR *P. aeruginosa* were not synergistic (Figure 2c, Table S7). In the case of the colistin-resistant *P. aeruginosa* strains, we observed additive effects or weaker synergistic effects (FIC index 0.31–0.75) but still observed 16-fold potentiation of moxifloxacin and 2- to 8-fold potentiation of ciprofloxacin at $^1/_4$ of the MIC of hybrid **1e** (Tables S5, S8). The reduced synergistic or additive effects of **1e** against colistin-resistant *P. aeruginosa* likely reflects reduced self-promoted uptake caused by transfer of cationic 4-amino-4-deoxy-L-arabinose to lipid A in these organisms.[18]

To gain insight into the structural requirements responsible for the observed adjuvant functions of hybrid **1e**, we also prepared two other tobramycin–C12–ciprofloxacin hybrids, the C-2″- and C-6″-tobramycin-linked ciprofloxacin hybrids **2** and **3** (Figure 1). The results confirmed that hybrids **2** and **3** are also able to synergize with ciprofloxacin and moxifloxacin for some XDR strains, thus suggesting that hybrid **1e** displays superior adjuvant properties (Tables S9, S10). To demonstrate that the synergistic effects of hybrid **1e** on the growth inhibition of *P. aeruginosa* translates into a measurable in vivo effect, we selected the *Galleria mellonella* infection model. *Galleria mellonella* larvae are an established in vivo model to study the efficacy of antimicrobial monotherapy[19,20] and combination therapy against MDR *P. aeruginosa*.[21,22] In pilot studies, we determined that **1e** causes $\leq 10\,\%$ hemolysis of human erythrocytes at $1000\ \mu g\,mL^{-1}$ (Figure S1 in the Supporting Information) and shows low cytotoxicity ($CC_{50} \gg 30\ \mu M$) against cancer cell lines (Figure S2). The maximal tolerated dose of **1e** by the larvae was found to be $150\ mg\,kg^{-1}$. Efficacy studies were performed by infecting the larvae with a lethal dose ($1.0 \times 10^3$ CFUs) of XDR *P. aeruginosa* #101856 followed by injection of the drug or drug combinations 2 h post infection. Monotherapy with a single dose of **1e** ($50\ mg\,kg^{-1}$) or moxifloxacin ($50\ mg\,kg^{-1}$) resulted in 100 % killing of the larvae within around 24 h, thus

  

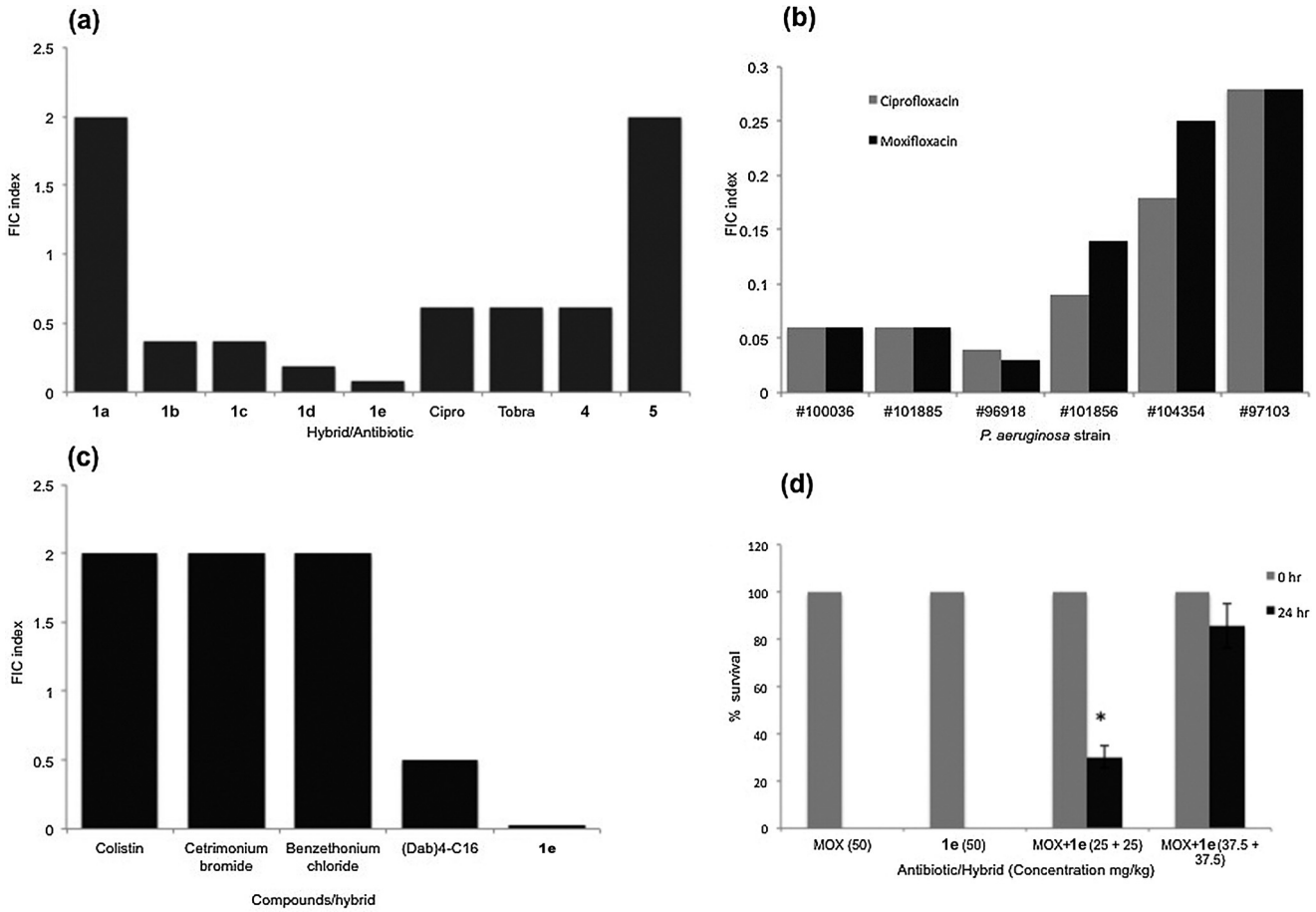


***Figure 2.*** a) Synergistic effects of hybrid antibiotics **1b**, **1c**, **1d**, and **1e** in combination with moxifloxacin in *P. aeruginosa* PAO1. In contrast, control compounds ciprofloxacin (Cipro), tobramycin (Tobra), amphiphilic tobramycin **4**, and amphiphilic ciprofloxacin **5** and hybrid **1a** showed additive effects with moxifloxacin. b) Synergistic effects of ciprofloxicin or moxifloxicin in combination with **1e** in MDR and XDR clinical isolates of *P. aeruginosa* (see Table S3). c) Combination studies on moxifloxacin with membrane-active cationic amphiphiles in XDR *P. aeruginosa* #96918. Colistin and cetrimonium bromide permeabilize the outermembrane of *P. aeruginosa* but do not synergize with moxifloxacin. Additive effects were also observed with benzethonium chloride and amphiphilic lipopeptide C16-Dab-Dab-Dab-Dab-$NH_2$ (Dab = L-2,4-diaminobutyric acid). d) Enhanced dose-dependent efficacy of a combination of **1e** and moxifloxacin in XDR *P. aeruginosa* (#101856) over a period of 24 h was demonstrated in a *Galleria mellonella* in vivo infection model. Combination therapy (25 mg $kg^{-1}$ **1e** + 25 mg $kg^{-1}$ moxifloxacin or 37.5 mg **1e** + 37.5 mg $kg^{-1}$ moxifloxacin) resulted in 30% or 86% survival of the larvae, respectively, after 24 h. In contrast, monotherapy with a single dosage of **1e** (50 mg $kg^{-1}$) or moxifloxacin (50 mg mg $kg^{-1}$) resulted in 100% killing of the larvae at $\leq$ 24 h. Each experiment involved **15** larvae from different batches; two experiments were performed in total per dosage of antibiotic/hybrid ($n = 30$). Significant differences between 0 and 24 h are indicated by * ($P$ value $\leq 0.05$).

indicating that monotherapy was not able to provide protection for the larvae. In contrast, combination therapy (25 mg **1e** + 25 mg $kg^{-1}$ moxifloxacin or 37.5 mg **1e** + 37.5 mg $kg^{-1}$ moxifloxacin) resulted in 30% or 86% survival of the larvae, respectively, after 24 h (Figure 2d). Higher dosages (75 mg **1e** + 75 mg moxifloxacin or 100 mg **1e** + 100 mg moxifloxacin) were able to protect 10% or 20% of the larvae for a 96 hour period, respectively, while monotherapy (100 mg $kg^{-1}$ **1e** or 100 mg $kg^{-1}$ moxifloxacin) resulted in 100% death after 36 h (Figures S3, S4). Although no further attempts to optimize the pharmacokinetics of the combination were performed, our results indicate short- and longer-term therapeutic potential for hybrid **1e**.

To gain insight into the protective function of **1e**, we performed a series of mechanistic studies with *P. aeruginosa* PAO1. First, we demonstrated that **1e** permeabilizes the outer membrane of *P. aeruginsosa* in a dose-dependent manner by using the NPN (1-*N*-phenylnaphtylamine) assay[23,24] (Figure 3a). We did not, however, observe significant depolarization of the cytoplasmic membrane as determined by using a depolarization assay[25] (Figure S5). Next, since **1e** is a conjugate of two different antibiotics, both of which are substrates for *P. aeruginosa* RND pumps,[26] we tested its role in altering the function of RND efflux pumps. We tested a MexAB-OprM deletion strain (PAO200), as well as an efflux-sensitive strain (PAO750)[27,28] that lacks five different clinically-relevant RND pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK, and MexXY) and the outer membrane protein OpmH, for potentiation of antibiotic activity by hybrid **1e**. We observed that **1e** was able to

  

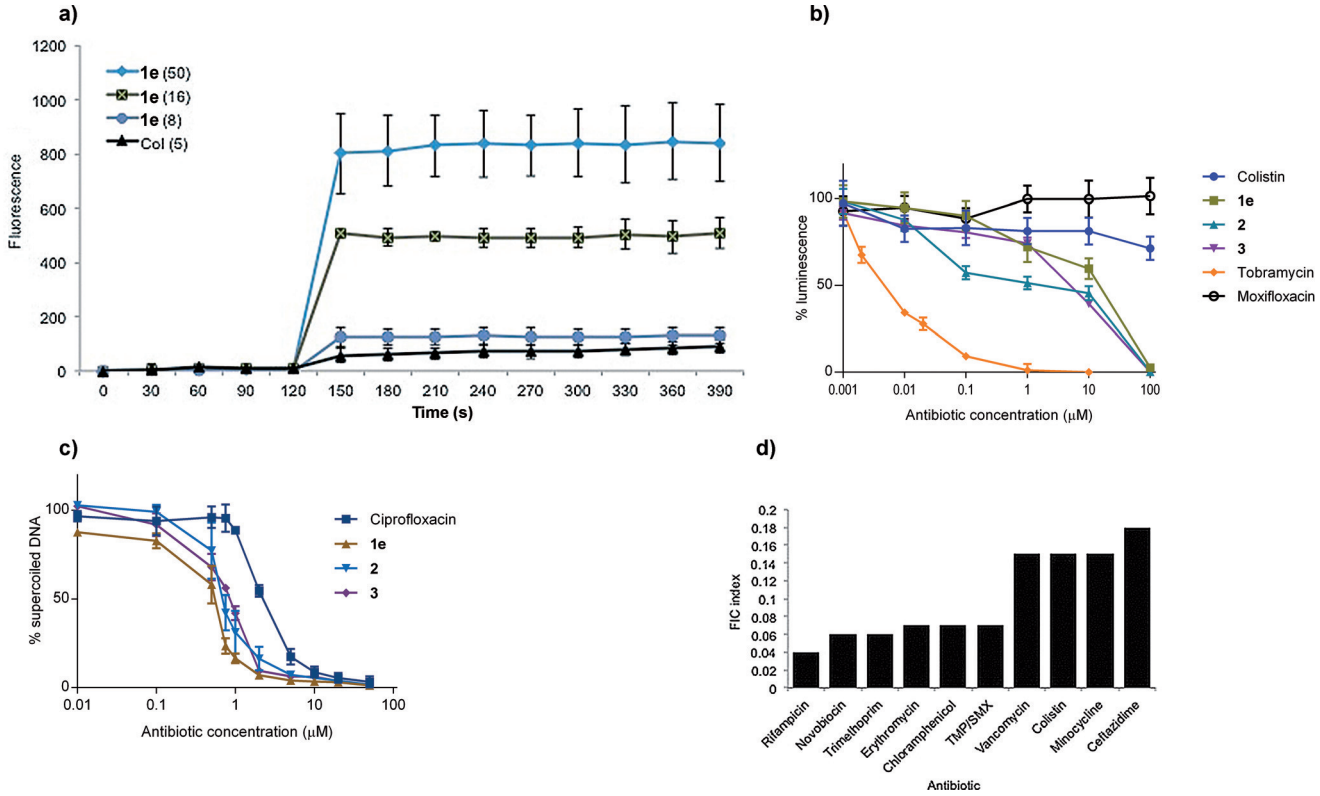


***Figure 3.*** a) Concentration-dependent permeabilization of the outer membrane by hybrid compound **1e** is indicated by the accumulation of 1-*N*-phenylnaphthylamine (NPN) in PAO1 cells. 50 μg mL$^{-1}$ (blue), 16 μg mL$^{-1}$ (tan), or 8 μg mL$^{-1}$ (purple) of **1e** were used, along with 5 μg mL$^{-1}$ colistin (black) as positive control. Compound **1e** was added at 120 seconds. b) Inhibition of in vitro protein translation in *E. coli* S30 extract by tobramycin, **1e**, **2**, and **3**. Protein translation assays were performed as described in Section S3.9 in the Supporting Information. The percentage luminescence was calculated compared to no antibiotic control (assumed to be 100%). Each data point represents an average luciferase activity of the triplicates with ± percentage error. The $IC_{50}$ values (μM) for tobramycin (0.0062 ± 0.0002), **1e** (8.05 ± 0.57), **2** (0.97 ± 0.05), and **3** (3.44 ± 0.09) were obtained as described in the Supporting Information. c) Inhibition of *P. aeruginosa* DNA gyrase A activity in the presence of ciprofloxacin, **1e**, **2**, or **3**. Each data point represents an average value of band (supercoiled) intensities from two independent sets of reactions (see Appendix S1 in the supporting information). The percentage of supercoiled DNA was measured compared to the no-antibiotic control (assumed to be 100%). $IC_{50}$ values (μM) for ciprofloxacin (2.4 ± 0.08), **1e** (0.5 ± 0.01), **2** (0.7 ± 0.03), and **3** (0.8 ± 0.02) were calculated (see Section S3.10 in the Supporting Information). d) Synergistic effects of **1e** with various classes of antibiotics according to the FIC index in PAO1.

potentiate the activity of moxifloxacin in both PAO200 (FIC, 0.12) and PAO750 (FIC < 0.5). Potentiation of antibiotic activity even in the absence of drug efflux pumps indicates that altering the activity of clinically-relevant RND pumps is not the mechanism by which hybrid **1e** potentiates the activity of antibiotics (Table S11). We also assessed whether the hybrids retain their original modes of action. In a protein translation assay, hybrids **2**, **3**, and **1e** showed a 156-fold, 554-fold, and 1290-fold reduction in activity, respectively, when compared to tobramycin (Figure 3b). In contrast hybrids **1e**, **2**, and **3** were 3–5-fold better inhibitors of DNA gyrase A (Figure 3c) and 2–3-fold better inhibitors of topoisomerase IV than ciprofloxacin (Figure S6).

The increased permeability of the outer membrane in PAO1 induced by hybrid **1e** is consistent with the strong synergy observed for this compound with outer membrane-impermeable antibiotics, including rifampicin (FIC index < 0.04), novobiocin (FIC index 0.06), trimethoprim (FIC index 0.06), erythromycin (FIC index 0.07), and chloramphenicol (FIC index 0.07). In addition, we were able to observe strong synergistic effects with polar antibiotics, including minocycline (FIC index 0.15) and ceftazidime (FIC index 0.18), but also colistin (FIC index 0.15). In contrast, hybrid **1e** did not demonstrate synergy with gentamicin (FIC index 2) or meropenem (FIC index 2; Figure 3d and Table S12). Combination studies of **1e** with moxifloxacin against other Gram-negative organisms (*E. coli* and *Acinetobacter baumannii*) indicated reduced synergistic effects (FIC index 0.25–0.36) compared to those seen in *P. aeruginosa*, while hybrids **2** or **3** showed additive effects (Table S13).

The discovery that the combination of tobramycin–ciprofloxacin hybrids with fluoroquinolone antibiotics overcomes resistance and enhances fluoroquinolone efficacy in XDR *P. aeruginosa* opens up new opportunities to develop therapies[29,30] against one of the most feared pathogens in hospitals and especially intensive care units. Moreover, our results suggest that tobramycin–ciprofloxacin hybrids are likely to enhance the efficacy of other antibacterials that suffer from poor penetration across the outer membrane of *P. aeruginosa.*

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